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Tebuconazole dissipation and metabolism in Tifton loamy sand during laboratory incubation†

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Abstract: The fungicide tebuconazole is widely used to control soil-borne and foliar diseases in peanuts and other crops. No published data are currently available on the extent and rate at which this compound degrades in soil. Unpublished data summarized in registration documents suggest that the compound is persistent, with 300–600 days half-life. We conducted a 63-day laboratory incubation to evaluate tebuconazole's dissipation kinetics and impact on soil microbial activity in Tifton loamy sand. Tifton soils support extensive peanut production in the Atlantic Coastal Plain region of Georgia and Alabama. Products containing tebuconazole are applied to an estimated 50% of the peanut acreage in the region. At the end of the incubation, 43 (±42)% of the parent compound was recovered in soil extracts. The first-order kinetic model, which gave a good fit to the dissipation data ($r^2 = 0.857$), yielded a soil half-life ($t_{1/2}$) of 49 days. This is 6–12 times more rapid than $t_{1/2}$ values described in unpublished tebuconazole registration documents. Four degradates were identified. Tentative structural assignments indicated that degradates were derived from hydroxylation of the parent compound and/or chlorophenyl ring cleavage. Cleavage products showed a steady increase during the incubation, and on a molar basis were equal to 63% of the time zero tebuconazole concentration. No significant effect on soil microbial biomass was observed, indicating that when the compound is applied at normal agronomic rate it does not impact soil metabolic activity. Use of the soil-half life data derived in this study should improve the accuracy of tebuconazole fate assessments for Coastal Plain peanut production. The study also indicated that environmental assessment of selected degradates may be needed to fully evaluate risks of tebuconazole use.
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Keywords: microbial biomass; respiration; degradation; fungicide; peanut

1 INTRODUCTION

Tebuconazole [(*RS*)-1-*p*-chlorophenyl-4,4-dimethyl-3-(1*H*-1,2,4-triazol-1-ylmethyl)pentan-3-ol] is used for disease control on fruit, nut, cereal and vegetable crops world-wide. Peanut producers in the Southeastern USA rely upon it heavily. The compound is highly effective in controlling soil-borne and foliar fungal pathogens, and has been credited with increasing yields above the levels provided by another widely used fungicide, chlorothalonil.[1–4] Data compiled by the USDA National Agricultural Statistics Service indicate that 41% of all USA peanut acreage was treated with tebuconazole during the 1999 growing season.[5] This is the most recent year for which data are available. Corresponding data for the state of Georgia, where US peanut production is centered, show that about 50% was treated. This accounted for 156 000 of the estimated 268 000 kg of active ingredient applied to peanuts annually.[5]

Although tebuconazole is intensively used, there are no published studies on soil degradation and/or dissipation rates. Unpublished degradation data summarized in registration documents and a risk assessment prepared by the working group of Joint FAO/WHO Meeting on Pesticide Residues (JMPR) have suggested that the compound is persistent, with aerobic soil metabolism half-lives ranging from 289 to 610 days.[1,6,7] It is not known how these data relate to environmental conditions in one of the compound's major use areas, the southern portion of the Atlantic Coastal Plain in the USA. The nation's peanut production is centered here and tebuconazole use is intense. To assess adequately the human and ecological risks of tebuconazole use and to evaluate the potential for residue accumulation in soil in the region, soil degradation studies are needed which reflect local conditions.

Data are also needed to assess impacts of tebuconazole on soil microbial activity. Several published

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studies have indicated that fungicide applications can alter community structure and depress or increase organic nitrogen mineralization rates, soil enzyme activities, microbial biomass and substrate-induced respiration.[8–12] No published studies have addressed how soil microbial communities respond to tebuconazole treatments. The compound's well-documented fungicidal activity and a recent report which found that tebuconazole was bactericidal in solution cultures of a *Mycobacterium* sp indicates that inhibition of soil microbial development and metabolic activity is possible.[13]

In this report, we describe tebuconazole dissipation kinetics, and accumulation and decay of selected degradates during laboratory incubation in Tifton loamy sand. Tifton soils support extensive peanut production in Coastal Plain regions in Georgia and Alabama.[14] Total microbial biomass carbon (MBC) and soil respiration (SR) were also monitored.

2 MATERIALS AND METHODS

2.1 Chemicals and supplies

Tebuconazole was purchased from Chem-Service (Chester, PA) and 2-chlorolepidine from Sigma-Aldrich (Milwaukee, WI). Optima® grade solvents, filters and other supplies were purchased from Fisher Scientific (Suwannee, GA).

2.2 Soil collection and preparation

A bulk sample of Tifton loamy sand (fine-loamy, siliceous, thermic, Plinthic Kaniudult, 1–2% slope) surface soil (top 2 cm) was collected from research plots located on a University of Georgia Research Farm near Tifton, Georgia (31°26′ N, 83°35′ W) in May 2002. Crops in the three previous years at the study site were: 1999—cotton, 2000—soybeans, 2001—cotton. No products containing tebuconazole had been used. The soil had the following characteristics: pH (H_2O) 6.1, total organic carbon (TOC) 0.41% and organic nitrogen (TON) 0.026%. Typical sand, silt and clay contents of Tifton surface soil are 910 g kg^{-1}, 40 g kg^{-1} and 50 g kg^{-1} respectively.[15] An 'at cracking' (emergence) application of the herbicide, metolachlor, was made at the study site about two weeks prior to collection of the soil sample.[15] Residues were measured in the soil by sequential extraction with methanol followed by high performance liquid chromatography-mass spectrometry. (HPLC-MS). The average concentration obtained from analysis of four sub-samples was 0.100 (±0.003) μg g^{-1}.

2.3 Laboratory incubations

Twenty-seven sub-samples (125 g) of soil that passed a #6 stainless steel sieve were placed in 250-ml French square glass bottles and brought to 13% water (by weight) with distilled-deionized water. Bottles were sealed with Teflon-lined screw caps. The water content was at the mid-point of the range of values reported for field capacity for Tifton surface soil.[14] After holding the samples at 24 °C for 6 days in a dark laboratory incubator, each bottle was fortified with 500 mg of quartz sand that had passed a #60 sieve. Bottles were shaken and returned to the incubator. The sand had been mixed with a solution of tebuconazole in methanol, the solvent allowed to evaporate, and the dry mixture blended with a stainless steel spatula. Tebuconazole was extracted from four 500-mg sand sub-samples with methanol and analyzed by HPLC with photodiode detection as described in Sections 2.6 and 2.7. The concentration was 220 (±2.2) μg g^{-1}. This translated to a tebuconazole treatment level of 0.9 μg g^{-1} soil. This is comparable with the label recommended application rate for individual treatments applied to peanuts if the active ingredient is uniformly mixed with the surface 1–2 cm of soil.[16] At weekly intervals, bottles were uncapped for 1 h to ensure aeration. Bottles were also weighed weekly and water lost was replaced using distilled-deionized water. At 0, 7, 14, 21, 28, 42 and 63 days, three randomly selected bottles were removed from the incubator. A sub-sample was collected from each for soil microbial biomass analysis. Methanol (50 ml) was then added to the bottles and they were stored at −10 °C until tebuconazole extraction and analysis was completed. Termination of the incubation at 63 days was based on the draft USEPA-OECD harmonized guideline for aerobic soil metabolism studies.[17]

2.4 Soil microbial biomass analysis

Soil microbial biomass (MBC) was estimated using a modification of the chloroform fumigation–extraction procedure.[18] A portion of each sub-sample (3 × 20 g) removed from each incubation bottle was extracted with aqueous potassium sulfate (0.5 M; 100 ml) by shaking for 1 h on a reciprocal shaker at 180 rev min^{-1}. Samples were then filtered (Whatman® GF-F) and the filtrate retained for analysis on a Shimadzu TOC 5000 DOC analyzer. The remaining soil was transferred to 20-ml glass scintillation vials to which ethanol-free chloroform (100 μl) was then added. To ensure even chloroform distribution throughout the sample, vials were placed in a vacuum desiccator with moist paper towels and a beaker containing ethanol-free chloroform (50 ml). The desiccator was evacuated three times to create a chloroform atmosphere and closed after the third evacuation. Samples were maintained in desiccators for 7 days at room temperature. They were then washed into 250-ml Erlenmeyer flasks with aqueous potassium sulfate (0.5 M; 100 ml) and extracted and analyzed for carbon as above. MBC was set equal to the difference in the mass of carbon extracted from soil with and without fumigation. An extraction efficiency of 0.35 as suggested by Voroney *et al*[19] was assumed.

2.5 Soil microbial respiration

Two samples (125 g) of soil were placed in incubation chambers of a constant volume respirometer (Comput-Ox 244, N-Con Systems, Crawford, GA)

and brought to 13% moisture using distilled-deionized water. The samples were incubated for 6 days at 24 °C. One sample was then fortified with the tebuconazole-treated sand as described in Section 2.3. The incubation chambers were reconnected to the respirometer and monitored for 63 more days. Data collection was at 30-min intervals.

2.6 Tebuconazole extraction

Bottles containing soil and methanol were brought to room temperature and placed on a bed-shaker operated at 210 rev min^{-1} for 30 min. The methanol was decanted through a glass-fiber filter (Whatman® GF/F) on a Buchner funnel support under vacuum. Two additional extractions were performed with 2×50 ml of methanol. After the third extraction, the soil was transferred to the Buchner funnel and rinsed with 2×10 ml methanol aliquots. The vacuum was maintained until the soil appeared dry. Combined filtrates were concentrated to 10 ml under a stream of high purity nitrogen gas. Extracts were syringe-filtered (0.45-μm PFTE® filters) and fortified with the internal standard, 2-chlorolepidine, at 5 μg ml^{-1}.

2.7 Extract analysis

Extracts were analyzed by HPLC linked in series to a photodiode array detector (PDA) and ion trap mass spectrometer using a Thermoquest LCQ® DECA system (Thermoquest-Finnegan, San Jose, CA). The mass spectrometer was operated with an atmospheric pressure chemical ionization (APCI) interface. The HPLC was fitted with a Beckman Ultrasphere® ODS column, 5 μm, 4.6 mm × 150 mm (Alltech, Deerfield, IL). Methanol (A) and 0.1% formic acid adjusted to pH 3.5 with ammonium hydroxide (B) were combined in the following gradient: initial conditions, 10% A/90% B hold 1 min, increase linearly to 90% A/10% B in 14 min, hold isocratic for 2 min, decrease to initial conditions in 1 min. The mass flow rate was 1 ml min^{-1}. Simultaneous scans of the PDA (190–300 nm) and mass spectrometer (m/z^+ 100–400) were made during the analysis. Before the analysis of each sample batch, or daily, mass spectrometer response was optimized on m/z^+ 308 while infusing a 10 μg ml^{-1} solution of tebuconazole in methanol into the HPLC column effluent with the mobile phase composition set at 80% A and 20% B. The ion m/z^+ 308 is the protonated tebuconazole parent $(M + H)^+$. All quantitation was based on a four-point calibration curve that was developed using solutions of tebuconazole in methanol over the concentration range 0.01–5 μg ml^{-1}. The r^2 values based on the mass spectrometer response for m/z^+ 308 was 0.999. Method limit of detection based on instrument response to the lowest calibration standard was 0.003 μg g^{-1}. Mass spectrum–mass spectrum (MS^2) analyses were conducted by in-source fragmentation of $(M + H)^+$ of each compound. Criteria used to classify chromatographic peaks as tebuconazole degradates included elution prior to tebuconazole, absence of the peak in extracts of untreated soil, and published HPLC-APCI-MS mass-spectral data of tebuconazole photo-oxidation products.[20] No degradate standards were available for confirmatory analysis or quantitation. Where quantitative data for degradates are reported, it was assumed that these compounds had similar extraction efficiencies and gave a detector response equivalent to the parent tebuconazole.

2.8 Quality control

All laboratory incubation samples were analyzed in triplicate. Among the seven sample sets analyzed, the relative standard deviation (RSD) averaged 26 (±29)%. Analysis of a series of matrix spikes prepared by addition of 50 μl of 1 μg ml^{-1} tebuconazole in methanol to 50 g of soil samples ($n = 3$), yielded RSD values of 5–14% based on MS and 7–8% on PDA response. The higher percentage RSD for incubated samples compared with matrix spikes was likely due to extraction of 'aged' (in incubated samples) versus 'fresh' residues in the matrix spikes and the fact that sub-samples were removed from incubated samples for MBC analysis. If tebuconazole were unevenly distributed in the soil, higher RSD values would result. Matrix spike and time-zero incubation sample data indicated that the extraction procedure provided quantitative tebuconazole recovery. Matrix recovery based on PDA detector response was 93 (±7.1)% and using MS data 117 (±5.7)%. High recovery based on MS detector response was probably due to matrix enhancement by co-extracted material from the soil. Matrix enhancements in HPLC-APCI-MS analysis of river water extracts have been reported for a number of pesticides.[21] It is anticipated that soil extracts would give the same type of enhancement. To account for it, quantitative results (based on MS data) for soil sample extracts were divided by 1.24. This factor was derived by comparison of PDA and MS matrix spike percentage recovery data. This computational adjustment normalized all results to PDA results. We chose to use the MS data and adjust for matrix effects as opposed to using PDA data because of enhanced MS specificity and sensitivity over PDA detection. In time-zero sample soils, the percentage recovery data after making the matrix response adjustment were unamended at 105 (±19)%. These data support the conclusion that tebuconazole was stable during frozen storage under methanol.

3 RESULTS AND DISCUSSION

3.1 Microbial biomass carbon

Microbial biomass carbon values fluctuated two to fourfold during incubations with and without tebuconazole, and followed a typical pattern of response to sample disturbance and re-wetting. Data are summarized in Fig 1. MCB ranged from about 100 μg g^{-1} on day 0 to about 200 μg g^{-1} on day 7, declining to about 50 μg g^{-1} after 21 days. It remained at this

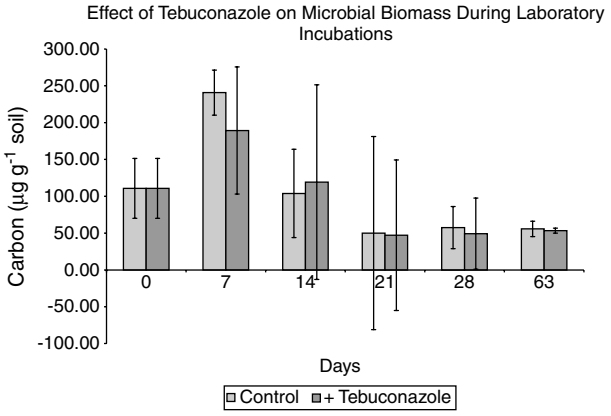

Figure 1. Effect of tebuconazole on soil microbial biomass carbon during laboratory incubation.

level in all subsequent measurements. Throughout the incubation period no significant differences between the tebuconazole-treated and the untreated samples were observed. Other studies have reported mixed responses of microbial biomass to pesticide treatment. For example, Harden *et al*[22] and Chen *et al*[8] reported reductions in microbial biomass following treatment with benomyl, isoproturon, simazine, dinoterb, chloroform, captan or chlorothalonil, while Hart and Brookes[23] reported no measurable effects following treatment with benomyl, chlorfenvinphos, aldicarb, triadimefon or glyphosate. Vieira *et al*[24] used the fumigation extraction technique to evaluate the impact of chlorothalonil on fungal biomass. These authors suggested that the lack of an observable response was due to the capacity for soil bacteria to respond rapidly to increased availability of carbon and nutrients derived from the biomass killed by the fungicide. A similar situation may be reflected in our studies, especially since our soils are so low in carbon.

3.2 Microbial respiration

As with microbial biomass, previous studies have provided mixed reports regarding the impact of pesticides on soil respiration. Tu[25] and Harden *et al*[22] reported increases in soil respiration following treatment with a wide range of pesticides while Anhalt *et al*[26] and Chen *et al*[8] demonstrated consistent reductions. It is interesting to note that some of the pesticides used in these studies were the same (benomyl and chlorothalonil), suggesting that the development of regional fate profiles for individual pesticides could provide information valuable to improved risk assessment. In our study, total microbial respiration was so low that it was not possible to determine whether tebuconazole treatment significantly affected overall microbial activity. Respirometric measurements showed that microbial respiration reduced soil TOC by only 80 and 30 $\mu g\,g^{-1}$ in the control and tebuconazole-treated soil, respectively, during the 63-day incubation period. We suggest that our soil has such a low organic carbon content that available carbon may not be sufficient to stimulate the soil microbial community enough to observe differences.

3.3 Tebuconazole dissipation and metabolite accumulation

Structures of the parent compound and proposed degradates are presented in Fig 2. MS and MS^2 data are summarized in Table 1. Degradates 2 $[(M+H)^+ = 224]$ and 4 $[(M+H)^+ = 168]$ correspond to KFE 1224 and STJ 5706 in the JMPR monograph.[1] Degradate 1 $[(M+H)^+ = 322]$ was recently described as a tebuconazole photooxidation product.[20] The structure proposed for degradate 3 has not previously been reported. It is the γ-hydroxy acid form of the γ-lactone, degradate 2. The prominent m/z^+ 194 in the MS^2 spectrum of degradate 3 supports its classification as a carboxylic acid. This ion likely resulted from a neutral loss of 46 (HCOOH). We also note that formation of γ-lactones from γ-hydroxy acids under acidic conditions and γ-lactone hydrolysis yielding the γ-hydroxy acid when treated with base are well known.[27] Some recent investigations reported co-occurrence of lactone and hydroxy acid metabolites in plasma in human drug metabolism studies.[28] Thus the co-occurrence of degradates 2 and 3 in soil incubations seems plausible.

Structures proposed in the JMPR monograph were linked to an unpublished report that is unavailable for independent review.[1] In the absence of these data, questions persist about the proposed structures, in particular, about degradate 2. Formation of degradate 2 requires nearly complete oxidation of the chlorophenyl ring of tebuconazole, leaving the carbon *para* to the chlorine atom for incorporation into the γ-lactone. The feasibility of such an oxidation by soil micro-organisms is unknown.

Data recently reported by Calza *et al*[20] suggest that degradate 2 may have an alternative elemental formula. During tebuconazole UV-irradiation in water

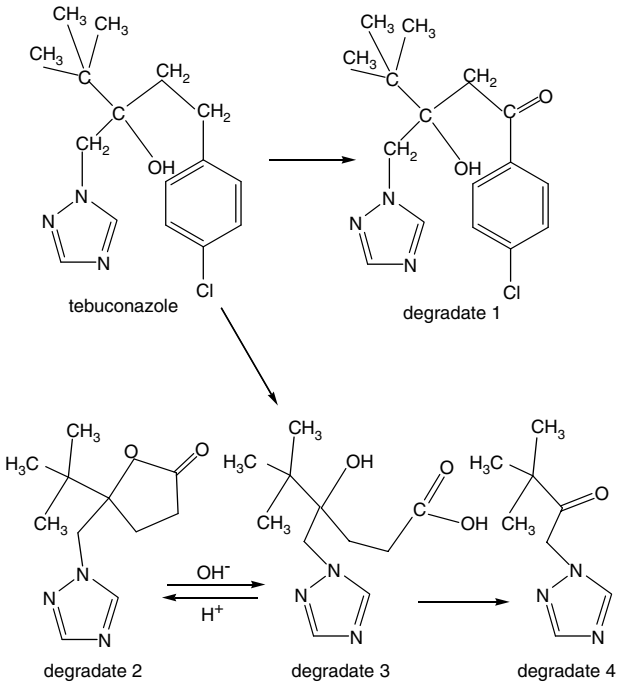

Figure 2. Structures of tebuconazole and proposed degradates.

Table 1. Mass spectral data for tebuconazole and selected degradates

Compound	$(M+H)^+$	Ion (m/z^+) and relative abundance (in parentheses)
Tebuconazole	308	MS: 308 (100), 310 (33), 311 (18), 309 (18), 290 (18) MS^2: 290 (56), 308 (69), 183 (35), 165 (100), 151 (99)
Degradate 1	322	MS: 322 (100), 323 (14), 324 (34), 325 (16), 260 (6), 239 (6) MS^2: 322(37), 304(100), 253 (70), 235 (11), 168 (66)
Degradate 2	224	MS: 224 (100), 225 (11), 223 (5), 114 (1) MS^2: 206 (100), 178 (10), 155 (10), 137 (37), 99 (13)1, 70 (87), 224 (3)
Degradate 3	240	MS: 240 (100), 241 (9) MS^2: 240 (71), 225 (55), 222 (100), 194 (40), 170 (26)
Degradate 4	168	MS: 168 (100), 169 (8), 166 (300, 140 (3) MS^2: 168 (100), 151 (3), 112 (3), 98 (4), 85(11)

they observed that a product with $(M+H)^+$ 322 accumulated and decayed, while there was steady accumulation of $(M+H)^+$ 224. Their MS and MS^2 data for tebuconazole and these products closely match our results for the parent compound and degradates 1 and 2. Calza *et al*[20] proposed $C_{10}H_{13}O_3N_3$ for $(M+H)^+$ 224. The formula for degradate 2 shown in Fig 2 is $C_{11}H_{17}N_3O_2$.

An additional ambiguity is that the product corresponding to $(M+H)^+$ 240 (degradate 3) was not reported in the photo-oxidation study mentioned above. This may be explained by a difference in instrumental conditions. In HPLC/APCI/MS analyses, we found that $(M+H)^+$ 240 abundance was highly sensitive to heated capillary temperature. When it was above 200 °C, this was not detected. Calza *et al*[20] reported that the capillary temperature was 220 °C in their analyses. Thus, the compound may have been present in their samples, but ions were degraded during transfer into the mass analyzer and not detected.

Taken together, available data do not support and or refute proposed structural assignments. Isolation of sufficient material to perform elemental analysis and or obtain IR and NMR spectra appears necessary for confirmation.

The mass accounting of degradates summarized in Table 2 shows that degradate 3 was most prominent, accounting for nearly 50% of the added tebuconazole when the incubation was terminated. This was followed by degradate 2 which accounted for about 15%. We note that the potential for pH-dependent inter-conversion between 2 and 3 makes relative percentages uncertain. Their summation is the best strategy when interpreting results. In this case, the data show that 61% of the added tebuconazole was present in the form of these degradates when the incubations were terminated. The other degradates, 1 and 4, were minor contributors accounting for <2%. We also note that in the terminal incubation samples, the sum of the tebuconazole and total degradates expressed as fraction of the initial tebuconazole concentration was 106%. Quantitative tracking of the parent and degradates and an internally consistent data set are indicated.

Finally, data compiled in Fig 3 show exponential tebuconazole decay and degradates accumulation. These data were fitted by linear regression to a first-order kinetic model. The r^2 values were 0.866 and

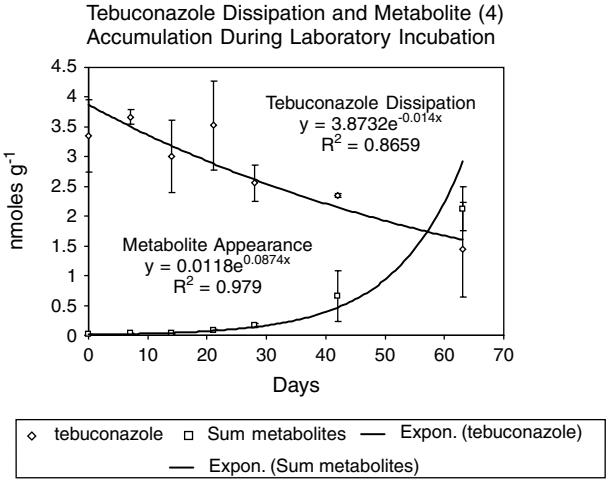

Figure 3. Changes in tebuconazole and metabolites concentration during incubation.

Table 2. Average tebuconazole and degradates concentration expressed as a percentage of moles of parent compound detected in Day-0 samples

	Tebuconazole	Degradate 1	Degradate 2	Degradate 3	Degradate 4	Total degradates
$(M+H)^+$	308	322	223	240	168	—
Day 0	100	0.29	0	0	0	0.29
Day 7	109	0.74	0.0	0.0	0.0	0.74
Day 14	89.5	0.85	0.0	0.0	0.0	0.85
Day 21	105	1.08	0.4	0.7	0.5	2.68
Day 28	76.3	0.77	1.1	2.4	0.7	4.97
Day 42	70.0	0.67	5.6	12.3	1.0	19.6
Day 63	42.9	0.52	14.9	46.5	1.4	63.3

0.979, respectively. The rate-constant obtained from the slope of the regression line yielded a tebuconazole soil half-life ($t_{1/2}$) of 49 days. This is 6–12 times faster than $t_{1/2}$ values described in tebuconazole registration documents.[1,6,7] We recently described relatively rapid dissipation in Tifton soil of other pesticides which, like tebuconazole, have relatively high soil organic carbon–water binding coefficients.[15,29] Contributing factors were relatively high temperature and moisture levels combined with low OC content. Low OC presumably resulted in low soil sorption and relatively high bioavailability.

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